



GALAR FUNGAIL STANDARD OPERATING PROCEDURES

GLASS SLIDE MICROARRAY PROTOCOL WITH “BALL-BUSTING” RNA PREP METHOD

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Adapted from the methods of the COGEME Consortium, UK

Preparation of Frozen Yeast Cell Drops

1. Grow 5 ml overnight cultures @ 30°C @ 200 rpm.
2. Grow cells to an OD of at least 0.5 in a volume of ~50-100 ml.
3. Split cultures into 2 Falcon tubes.
4. Centrifuge for 3 min @ 4,000 rpm.
5. Pour off as much supernatant as possible
6. Resuspend pellets in the remaining supernatant (~200 µl in total).
7. Pre-cool 2 screw-capped Eppendorfs in liquid N₂.
8. Carefully release small drops of cell suspension into the liquid N₂ in the Eppendorfs (GOGGLES ON!). Try to do this slowly so that the drops don't merge too much – not easy!
9. Frozen cell drops can be stored at –80°C or continue with RNA isolation straight away.

ISOLATION OF TOTAL RNA

1. Soak a 5 ml Teflon vessel and 7 mm tungsten carbide bead in RNase Away, then pre-cool in liquid N₂
2. Add the frozen cell drops and the bead to the Teflon vessel and screw shut.
3. Insert into the Micro-Dismembrator. Shake for 2 min @ 1200 rpm.
4. Ensure that there are no un-powdered lumps of cells left and resuspend immediately in 2 ml Trizol (phenol - VERY TOXIC).
5. Transfer into 2 Eppendorfs. Vortex for 1 min.
6. Leave for 5 min @ room temperature to allow for dissociation of the nucleoprotein complexes.
7. Centrifuge for 10 min @ 12,000 g.
8. Tip supernatant into a new Eppendorf.
9. Add 0.4 vol chloroform. Shake samples vigorously by hand for 15 s.
10. Leave for 3-10 min @ room temperature.
11. Centrifuge for 5 min @ 12,000 g.
12. Transfer the top (colourless) layer into a new Eppendorf avoiding the interphase.
13. Add 0.5 vol isopropanol. Precipitate DNA for 5-15 min @ room temperature.
14. Centrifuge for 10 min @ 12,000 g. Carefully remove the supernatant.
15. Wash the pellet with 1 ml ice-cold 70% ethanol. Vortex.
16. Centrifuge for 10 min @ 12,000 g. Carefully remove the supernatant.
17. Briefly air dry the RNA pellet / Speed-Vac for 1 min.
18. Resuspend the pellet in 500 µl DEPC-treated H₂O. Vortex.
19. Add 500 µl LiCl buffer. Vortex.
20. Leave to precipitate @ -20°C for at least 1 h or overnight.
21. Centrifuge for 30 min @ max.
22. Wash the pellet with 1 ml 70% ethanol. Centrifuge for 10 min @ max.
23. Wash the pellet with 500 µl 70% ethanol. Centrifuge for 10 min @ max.
24. Remove ethanol with a pipette.
25. Air dry pellet for ~15 min / Speed Vac for 5 min.
26. Resuspend in ~50 µl DEPC H₂O.
27. Run 1 µl on a 1% TBE-agarose gel to visualise and check for RNA integrity.
28. Take the OD₂₆₀ of 1/1000 dilution to quantify the RNA.

$$\text{OD}_{260} \text{ of } 1.0 = 40 \mu\text{g } \mu\text{l}^{-1}$$

$$\text{therefore } [\text{RNA}] = \text{OD}_{260} \times 40 \times 1000 / 1 \times 1000 = \text{OD}_{260} \times 40 \mu\text{g } \mu\text{l}^{-1}$$

PREPARATION & PURIFICATION OF LABELLED CDNA

1. Prepare 300 μ l labelling master mix as follows (n=20). Store unused @ -20°C:

110.4 μ l	DEPC H ₂ O	
120 μ l	5x RT buffer	(1x)
60 μ l	5 mM DTT	(1 mM)
3 μ l	100 mM dATP	(500 μ M)
3 μ l	100 mM dCTP	(500 μ M)
3 μ l	100 mM dGTP	(500 μ M)
0.6 μ l	100 mM dTTP	(100 μ M)
2. To 50 μ g RNA add 2 μ g oligo dT (4 μ l of 0.5 μ g μ l⁻¹). Make up to 10 μ l with DEPC H₂O.
3. Incubate for 10 min @ 70°C.
4. Incubate for 1 min on ice.
5. Add 15 μ l of the labelling master mix.
6. Add 3 μ l Cy3 dUTP (Amersham) to one sample and 3 μ l Cy5 dUTP (Amersham) to the other sample (to which the first is to be compared).
7. Add 2 μ l Superscript II reverse transcriptase (Invitrogen; 400 U).
8. Mix thoroughly. Pulse to collect the contents at the bottom of the tube.
From now on keep the samples hidden from light as much as possible.
9. Incubate for 2 h @ 42°C.
10. Pulse to collect the contents at the bottom of the tube.
11. Add 1.5 μ l 20 μ M EDTA to stop the reaction.
12. Add 1.5 μ l 500 mM NaOH. Incubate for 10 min @ 70°C to degrade the RNA.
13. Add 1.5 μ l 500 mM HCl to neutralise the reaction.
14. Add 500 μ l of Capture Buffer to the purification column.
15. Add 34.5 μ l of a Cy3 and of a Cy5 sample (co-purify samples which will be co-hybridised). Mix.
16. Centrifuge for 30 s @ max. Discard contents of collection tube.
17. Add 500 μ l Wash Buffer (with ethanol added).
18. Centrifuge for 30 s @ max. Discard collection tube.
19. Transfer column into a fresh Eppendorf.
20. Add 50 μ l Elution Buffer or sterile H₂O. Leave for 1 min @ room temperature.
21. Centrifuge for 1 min @ max. Discard column.
22. Run 3 μ l on a 1% TBE-agarose gel and scan with Phospho-imager @ 633 nm (Cy5).
23. Dry in Speed Vac (~15 min). Resuspend to 20 μ l.

HYBRIDISATION OF GLASS SLIDE MICROARRAYS

Prehybridisation:

1. Make 100 ml Prehybridisation Mix containing:

25 ml	20x SSC	(5x)
1 ml	10% SDS	(1%)
1 g	BSA	(1%)
2. Insert the slides in the above mix in a Coplin jar ensuring that they are not touching!
3. Prehybridise for 45 min @ 42°C.
4. Wash by running under dH₂O tap. Tip out and refill 5 times.
5. Fill jar with isopropanol.
6. Air dry slides.

Hybridisation:

7. Make 10.2 ml 2x Hybridisation Mix containing:

5 ml	formamide	(50%)
5 ml	20x SSC	(10x)
0.2 ml	10% SDS	(0.2%)
8. Add 20 µl of this to 20 µl of the purified Cy3- and Cy5-labelled cDNA mix.
9. Boil for 3 min to denature. Pulse to collect contents at the bottom of the tube.
10. Carefully add the mixture to the middle of the printed part of the microarray slide.
11. Place a 22x50 mm cover-slip over the mixture. Bubbles should resolve themselves.
12. Place in a hybridisation chamber.
13. Incubate overnight @ 42°C.

Washing:

14. Prepare washing buffers.
15. Wash slides in 2x SSC, 1% SDS for 15 min @ room temperature with agitation.
16. Wash slides in 1x SSC, 0.2% SDS for 8 min @ room temperature with agitation.
17. Wash slides in 0.1x SSC, 0.2% SDS for 5 min @ room temperature with agitation.
18. Place slide in a 50 ml Falcon tube. Centrifuge for 4 min @ 3.5K to dry.
19. Scan slide.

GENERAL BUFFERS

1 M TRIS.HCl	200 ml	500 ml	1000 ml
Tris	24.22 g	60.55 g	121.1 g
dH ₂ O	160 ml	400 ml	800 ml

Adjust pH with conc HCl:

pH 7.4 14 ml 35 ml 70 ml

pH 7.6 12 ml 30 ml 60 ml

Make up to full volume with dH₂O.

Autoclave.

0.5 M Na₂EDTA	100 ml	200 ml	500 ml	dH₂O
Na ₂ EDTA (MW: 372.08)	18.6 g	37.2 g	93.05 g	

pH to 8.0 to ensure solubility

ORDER NUMBERS

Dyes: Cy3 dUTP	Amersham	PA53022
Cy5 dUTP	Amersham	PA55022
Oligo(dT) primer:		
Oligo(dT) ₁₅ (500 µg/ml)	Promega	C110A
Reverse Transcriptase:		
Superscript II (RNaseH minus)	Invitrogen	18064-022

SOLUTIONS FOR RNA PREPS & cDNA SYNTHESIS

Ensure weighing is done without spatulas, mark all solutions RF and don't touch without gloves.

DEPC TREATED H₂O	200 ml	500 ml	1000 ml	dH₂O
Diethyl polycarbonate(0.1%)	0.2 ml	0.5 ml	1 ml	
Leave overnight. Autoclave.				
70 % ETHANOL	100 ml	200 ml	500 ml	
RNase Free ethanol	70 ml	140 ml	350 ml	
DEPC treated H ₂ O	30 ml	60 ml	150 ml	
4 M LITHIUM CHORIDE	100 ml	200 ml	500 ml	DEPC H₂O
LiCl (MW = 42.4)	16.96 g	33.92 g	84.8 g	
LITHIUM CHLORIDE BUFFER	20 ml	50 ml	100 ml	
4 M LiCl (4 M)	20 ml	50 ml	100 ml	
1 M Tris.HCl pH 7.4 (20 mM)	4 µl	10 µl	20 µl	
0.5 M EDTA (10 mM)	4 µl	10 µl	20 µl	
20 mM EDTA	20 ml	50 ml	100 ml	
0.5 M Na ₂ EDTA	0.8 ml	2 ml	4 ml	
DEPC treated H ₂ O	19.2 ml	48 ml	96 ml	
500 mM NAOH	5 ml	10 ml	20 ml	DEPC H₂O
NaOH (MW = 40)	0.1 g	0.2 g	0.4 g	
500 mM HCL	5 ml	10 ml	20 ml	
11.6 M HCl (from supplier)	215.5 µl	431 µl	862 µl	
DEPC treated H ₂ O	4.785 ml	9.569 ml	19.138 ml	

HYBRIDISATION & WASHING SOLUTIONS FOR MICROARRAYS

Ensure weighing is done without spatulas, mark all solutions RF and don't touch without gloves.

20x SSC	200 ml	500 ml	1000 ml	DEPC H₂O
NaCl (3 M) (MW = 58.4)	35.04 g	87.6 g	175.2 g	
Na ₃ Citrate.2H ₂ O (0.3M) (MW = 294.1)	17.65 g	44.12 g	88.23 g	
10% SDS	100 ml	200 ml	500 ml	DEPC H₂O
SDS (MW = 288.4)	10 g	20 g	50 g	
PREHYBRIDISATION BUFFER	100 ml	200 ml	500 ml	
20x SSC (5x)	25 ml	50 ml	125 ml	
10% SDS (0.1%)	1 ml	2 ml	5 ml	
BSA (1%)	1 g	2 g	5 g	
dd H ₂ O	74 ml	148 ml	370 ml	
2x HYBRIDISATION BUFFER	2 ml	5 ml	10 ml	
20x SSC (10x)	1 ml	2 ml	5 ml	
10% SDS (0.2%)	1 ml	2 ml	5 ml	
Formamide (50%)	40 µl	100 µl	200 µl	
WASH BUFFER I	50 ml	100 ml	200 ml	
20x SSC (2x)	5 ml	10 ml	20 ml	
10% SDS (1x)	5 ml	10 ml	20 ml	
dd H ₂ O	40 ml	80 ml	160 ml	
WASH BUFFER II	50 ml	100 ml	200 ml	
20x SSC (1x)	2.5 ml	10 ml	20 ml	
10% SDS (0.2x)	1 ml	2 ml	4 ml	
dd H ₂ O	46.5 ml	88 ml	176 ml	
WASH BUFFER III	50 ml	100 ml	200 ml	
20x SSC (0.1x)	0.25 ml	0.5 ml	1 ml	
10% SDS (0.2x)	1 ml	2 ml	4 ml	
dd H ₂ O	48.75 ml	97.5 ml	195 ml	